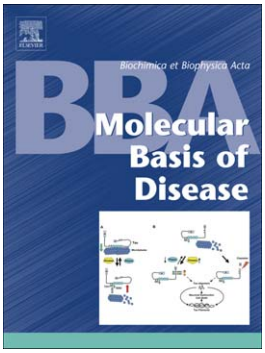

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Cystathionine beta synthase deficiency induces catalase-mediated hydrogen peroxide detoxification in mice liver

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Abstract

Cystathionine beta synthase deficiency induces hyperhomocysteinemia which is considered as a risk factor for vascular diseases. Studies underlined the importance of altered cellular redox reactions in hyperhomocysteinemia-induced vascular pathologies. Nevertheless, hyperhomocysteinemia also induces hepatic dysfunction which may accelerate the development of vascular pathologies by modifying cholesterol homeostasis. The aim of the present study was to analyze the modifications of redox state in the liver of heterozygous cystathionine beta synthase-deficient mice, a murine model of hyperhomocysteinemia. In this purpose, we quantified levels of reactive oxygen and nitrogen species and we assayed activities of main antioxidant enzymes. We found that cystathionine beta synthase deficiency induced NADPH oxidase activation. However, there was no accumulation of reactive oxygen (superoxide anion, hydrogen peroxide) and nitrogen (nitrite, peroxynitrite) species. On the contrary, hepatic hydrogen peroxide level was decreased independently of an activation of glutathione-dependent mechanisms. In fact, cystathionine beta synthase deficiency had no effect on glutathione peroxidase, glutathione reductase and glutathione S-transferase activities. However, we found a 50% increase in hepatic catalase activity without any variation of expression. These findings demonstrate that cystathionine beta synthase deficiency initiates redox disequilibrium in the liver. However, the activation of catalase attenuates oxidative impairments.

Introduction

Homocysteine (Hcy) is a thiol-containing amino acid at the crossroad of the methionine metabolism (Fig. 1). Dietary methionine is adenosylated by methionine adenosyl transferase to form S-adenosyl methionine, the universal methyl donor. Once formed via the demethylation pathway, Hcy may undergo remethylation to form methionine via folate- or betaine-dependent mechanisms. Otherwise, Hcy may enter the transsulfuration pathway leading to the formation of cysteine and then glutathione (GSH), the major cellular antioxidant. GSH is used by glutathione peroxidase (GPx) to detoxify hydrogen peroxide (H_2O_2) and oxidized glutathione (GSSG) is subsequently regenerated to GSH by glutathione reductase (GRed). GSH also serves as a substrate for glutathione S-transferase (GST) in the detoxification of xenobiotics or endogenous compounds such as lipid peroxides.

Abnormally high plasma Hcy levels are characteristic of hyperhomocysteinemia, a metabolic disease which can be due to altered genetic or nutritional factors involved in Hcy metabolism [1]. Three different types of hyperhomocysteinemia were identified depending on Hcy concentration: moderate (15-30 µM), intermediate (30-100 µM) and severe (> 100 µM) [2]. Necropsies of severe hyperhomocysteinemic patients notably revealed atherosclerosis, thrombosis and hepatic steatosis [3,4]. Moreover, even a moderate elevation of total Hcy level is recognized as an independent vascular risk factor for atherothrombosis in the coronary, cerebrovascular and peripheral arterial circulation [5-8].

Several studies reported impaired endothelial functionality and antithrombotic mechanisms [9]. In vivo studies supported by in vitro results imputed notably the impairment of endothelial vasomotor function to Hcy-induced reduced NO bioavailability [10,11]. Even if vascular toxicity of Hcy is indisputable, the hepatic

dysfunction might play a key role in the development of vascular diseases. In this sense, Hcy metabolism is mainly hepatic [12] and liver damage promotes hyperhomocysteinemia through cystathionine beta synthase (CBS), betaine homocysteine methyl transferase and methionine synthase downregulation [13]. Furthermore, several vascular atherogenic factors arise from hepatic dysfunction induced by hyperhomocysteinemia [14-18]. In vitro studies have shown that cellular damage induced by Hcy were linked to oxidative stress [19, 20] and GPx-1 overexpression rescued Hcy-induced endothelial dysfunction in vivo [21]. Hcy is thought to induce reactive oxygen species (ROS) accumulation either by direct ROS generation via auto-oxidation [22] or by disturbing the balance between prooxidant and antioxidant activities. Such a desequilibrium between ROS producing and detoxifying enzymatic activities arises primarily from an increased production of superoxide anion ($O_2^{\cdot -}$), the univalent reduction of dioxygen, by mitochondrial respiratory complexes or various oxidases such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase. $O_2^{\cdot -}$ can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a DNA and protein damaging agent. Thus an increase in $O_2^{\cdot -}$ production can decrease NO bioavailability. Therefore $O_2^{\cdot -}$ is rapidly dismutated into H_2O_2 by superoxide dismutase (SOD). However, H_2O_2 itself is a cytotoxic oxygen radical and leads through the Fenton's reaction to the formation of the highly reactive hydroxyl radical ($HO^{\cdot}$). Thus H_2O_2 is degraded to H_2O and O_2 by catalase (CAT) and GPx.

Experimental hyperhomocysteinemia can be obtained by dietary interventions such as vitamin B_6, B_{12},and folate deficiency or methionine supplementation, or by genetic approaches such as CBS [23], methylene tetrahydrofolate reductase [24] and methionine synthase [25] deficiency. Some of these hyperhomocysteinemic models

have notably been used to elicit Hcy influence on main hepatic antioxidant activities [26-31]. However, the influence of CBS deficiency on hepatic redox state has never been extensively studied. Heterozygous CBS-deficient mice show hepatic gene expression modulation of several cytochromes, indicating perturbation of the hepatic redox potential [32]. Therefore, we characterized the hepatic redox state in CBS-deficient mice by quantification of ROS, reactive nitrogen species (RNS) and activities of the main enzymes involved in redox homeostasis.

Materials and Methods

Chemicals and antibodies

Unless mentioned otherwise all chemicals and antibodies were obtained from Sigma (Sigma-Aldrich, Saint Quentin Fallavier, France).

Mice

Animal care was conducted in accordance with internal guidelines of the French Ministère de l'Agriculture for animal handling. Mice heterozygous for targeted disruption of the *Cbs* gene (Cbs +/-) [23] were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA). Cbs +/- mice, on a C57BL/6 background, and their wild-type C57BL/6 littermates (Cbs +/+) were used. Cbs +/- mice were produced by mating male Cbs +/- mice with female Cbs +/+ mice. Tail biopsies were performed on mice at 4 weeks of age and polymerase chain reaction was used for genotyping as previously described [23]. Mice were fed a standard laboratory diet (A03, Safe, Augy, France) given *ad libitum*. This diet has an approximative protein content of 21%, a methionine content of 4.8 $g.kg^{-1}$, a folic acid content of 0.9 $mg.kg^{-1}$ and a vitamin B_6 and B_{12} content of respectively 3.5 and 0.04 $mg.kg^{-1}$.

Preparation of samples and plasma total Hcy assay

At time of sacrifice, blood samples were collected into tubes containing 1/10 volume of 3.8% sodium citrate and placed on ice immediately. Plasma was isolated by centrifugation at 2500 g for 15 min at 4°C. Plasma total Hcy was assayed by using the fluorimetric high-performance liquid chromatography method described by Fortin and Genest [33]. Livers were harvested, snap-frozen and stored at -80°C until use.

100 mg of liver were homogenized in 500 µL Dulbecco's phosphate-buffered saline (Invitrogen, Cergy, France) containing proteases inhibitors. Homogenates were centrifuged at 13000g for 15 min at 4°C. Supernatants were then assayed for protein concentrations by the Bradford assay.

RNA extraction and determination of mRNA levels

RNA were prepared from liver with the NucleoSpin RNA II kit (Macherey Nagel, Hoerdt, France). Quantity and purity of the RNA were assessed by measuring absorbance at 260 and 280 nm. Reverse transcription was carried out on 1 µg RNA as described by the manufacturer (Applied Biosystems, Courtaboeuf, France). Catalase and heme oxygenase 1 (HO-1) mRNA levels were analyzed by real-time quantitative reverse transcription-polymerase chain reaction as previously described [18] using the Light Cycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Meylan, France). Primers (Eurogentec SA, Seraing, Belgium; Table 1) were designed with the Primer3 software [34] to yield a single amplicon based on dissociation curves. Each reaction was performed in duplicate. The mouse superoxide dismutase-1 mRNA was used as an endogenous control [32]. ΔΔCp analysis of results allows to assess the ratio of the target mRNA versus control mRNA [35].

Western Blot analysis

60 µg of total proteins were subjected to SDS electrophoresis on 12% acrylamide gels under reducing conditions and transferred to Hybond-C Extra membrane (GE Healthcare Europe GmbH, Saclay, France). After transfer, membranes were blocked in 10% nonfat dry milk in Tris-saline buffer (1.5 mM Tris, 5

mM NaCl, 0.1% Tween 20) and probed with anti-heme oxygenase 1 (HO-1) antibody (Calbiochem, San Diego, USA). Horseradish peroxidase-conjugated secondary antibody and Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Heidelberg, Germany) were used to detect specific proteins. β-actin was used as internal control.

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured by monitoring the oxidation rate of cytochrome c as described by Iwai *et al.* [36] with 20 µg of total proteins. NADPH oxidase activity was quantified from the absorbances at 540, 550 and 560 nm as previously described [37].

Determination of superoxide anion content

$O_2^{\cdot -}$ levels were determined on 300 µg of total proteins after hemoglobin precipitation by monitoring the oxidation of dihydroethidium into ethidium as described by Woo *et al.* [26].

Measurement of SOD activity

SOD activity was measured with 20 µg of total proteins by using the Superoxide Dismutase Kit (R&D Systems Europe, Lille, France) following supplier's instructions. Measurement of enzyme activity is based on the competition of SOD and nitroblue-tetrazolium for $O_2^{\cdot -}$.

Determination of hydrogen peroxide content

H_2O_2 was quantified on 100 µg of proteins, after hemoglobin precipitation, with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Cergy, France) as recommanded by supplier's instructions. Measurement of H_2O_2 level is based on the production of resofurin by H_2O_2 and Amplex Red reagent in the presence of peroxidase.

Determination of nitrites and nitrates contents

Nitrites and nitrates levels were determined by Marzinzig *et al.* modified Griess reaction [38]. 8 mg of total protein were deproteinized by acetonitrile-chloroform protein precipitation [39]. To reduce nitrates into nitrites 400 µL of deproteinized sample were incubated in the dark for 1 hour at 37°C with 20 µL FAD 200 µM, 20 µL NADPH 6 mM and 10 µL nitrate reductase 5 U/mL. Then excess NADPH was oxidized with 20 mM sodium pyruvate and 0.5 U L-lactate dehydrogenase in a final volume of 500 µL. Total nitrites levels (representing nitrites and nitrates) were spectrophotometrically determined at 550 nm after 5 minutes incubation with not premixed 250 µL ice-cold dapsone (4,4'-diamino-diphenylsulfone; 14 mM in 2 N HCl) and 250 µL 4 mM N-(1-naphtyl)-ethylendiamine.

Determination of peroxynitrite content

$ONOO^-$ levels were measured by assaying $ONOO^-$-mediated nitration of phenol with 200 µg of total proteins as described by VanUffelen *et al.* [40].

Measurement of catalase activity

CAT activity was measured on liver extract corresponding to 25 µg of total proteins in 1.5 mL of 50 mM Tris buffer pH 7.8, containing 2 mM $MgCl_2$ and 2 mM

$CaCl_2$.The maximum CAT activity was determined with a sensitive Clark electrode measuring dioxygen produced by the dismutation of H_2O_2. 2-8 µL H_2O_2 (100 mM) were progressively injected, in order to obtain the maximum production rate of dioxygen.

Measurement of glutathione peroxidase activity

GPx activity was measured by the modified Paglia and Valentine method [41] described by Carmagnol *et al.* [42]. Hemoglobin was first converted to cyanmethemoglobin to avoid interference. Total proteins were incubated at 30°C in 1 mL of 0.1 M potassium phosphate buffer with 1 mM glutathione, 1mM tert-butyl hydroperoxide, 1.4 units of GRed (Type III), 1.43 mM NADPH and 1.5 mM KCN. GSSG produced by GPx and hydroperoxide was reduced by NADPH and GRed. Then NADPH oxidation was followed spectrophotometrically at 340 nm.

Measurement of glutathione reductase activity

GRed activity was measured by spectrophotometrically monitoring NADPH oxidation at 340 nm as described by Carlberg and Mannervik [43]. Total proteins were incubated at 30 °C in 1 mL of 0.1 M potassium phosphate buffer with 2 mM NADPH and 20 mM glutathione disulfide.

Measurement of glutathione S-transferase activity

GST activity was assayed by the method described by Habig *et al.* [44]. S-conjugate formation was followed at 25°C, pH 6.5 in a 0.1 M phosphate potassium buffer with 1mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene by monitoring changes in absorbance at 340 nm.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was done with Mann-Whitney-Wilcoxon's test using R software (http://www.R-project.org). Data were considered significant when $p \leq 0.05$.

Results

Plasma Hcy levels in CBS-deficient mice

In order to characterize the effects of CBS deficiency on hepatic redox status, we used male Cbs +/- mice and male Cbs +/+ from the same litter aged from 6 to 10 months. As expected, heterozygous CBS-deficient mice displayed abnormal plasma Hcy levels compared to wild type mice (8.9±0.8 µM, n=10 vs. 3.9±0.3 µM, n=8; $p<0.0001$).

HO-1 expression in the liver of CBS-deficient mice

Induction of HO-1 expression is recognized as a marker of oxidative stress [45]. Therefore, the hepatic expression of HO-1 was analyzed to determine if the rise of Hcy induced by heterozygous CBS deficiency was sufficient to induce oxidative stress. We found an overexpression of HO-1 mRNA in the liver of Cbs +/- mice (100.0±6.0 % vs. 146.4±15.2 %; $p=0.015$, Fig. 2A). In agreement with increased levels of HO-1 mRNA, albeit the difference was not significant, HO-1 protein expression appeared to be increased (100.0±26.7 %, n=4 vs. 159.0±24.1 %, n=4; $p<0.1$, Fig. 2B).

Effect of CBS deficiency on hepatic ROS production

Even though NADPH oxidase activity was increased in liver of Cbs +/- mice (100.0±2.0 % vs. 109.6±2.5 %, $p=0.04$, Fig. 3A), there was no increase in $O_2^{\cdot -}$ level. On the contrary, we observed a non significant decreased concentration of $O_2^{\cdot -}$ (100.0±10.1 % vs. 81.1±5.6 %, $p=0.28$, Fig. 3B). The regulation of hepatic level of $O_2^{\cdot -}$ could be due to an activation of SOD. However, we only observed a non significant increase in SOD activity (100.0±7.8 % vs. 113.1±7.1 %, $p=0.48$, Fig. 3C)

and surprisingly, we found a significant decrease in H_2O_2 level in liver of Cbs +/- mice compared to Cbs +/+ mice (100.0±5.2 % vs. 75.8±10.3 %, p=0.04, Fig. 3D).

Effect of CBS deficiency on hepatic RNS production

Reduced NO bioavailability is a key process in Hcy-induced vascular pathology but the implication of NO in liver diseases remains unclear [46]. We measured nitrites and nitrates levels as being representative of NO release. Measurement of nitrites alone did not gave detectable signal indicating that, in the liver, nitrates are the main NO derivatives. After nitrates reduction into nitrites we did not observed any significant difference between hepatic concentrations of Cbs +/- and Cbs +/+ mice (100.0±4.8 % vs. 104.0±8.5 %, p=0.80, Fig. 4A). The reaction of $O_2^{\cdot -}$ with NO leads to the formation of $ONOO^-$. As we have observed an increased production of $O_2^{\cdot -}$ by NADPH oxidase but no accumulation of $O_2^{\cdot -}$, this could be due to an immediate reactivity of $O_2^{\cdot -}$ with NO. However, there was no increase in $ONOO^-$ generation (100.0±12.6 % vs. 91.5±7.7 %, p=0.70, Fig. 4B) in liver of Cbs +/- mice compared to Cbs +/+ mice.

Effect of CBS deficiency on hydrogen peroxide detoxification

As we have observed a decreased H_2O_2 hepatic content, we investigated by which mechanism this ROS was detoxified. GPx is able to detoxify H_2O_2 by oxidizing GSH, the main cellular antioxidant compound. So we assayed GPx and GRed to determine if the decrease in H_2O_2 could be due to an activation of the GSH-dependent detoxification cycle. Both GPx (100.0±4.3 % vs. 105.7±5.1 %, p=0.37, Fig. 5A) and GRed (100.0±3.0 % vs. 103.9±4.2 %, p=0.50, Fig. 5B) activities remained unchanged in liver of Cbs +/- mice compared to Cbs +/+ mice. We also assayed

hepatic GST activity (100.0±6.8 % vs. 95.4±12.5 %, p=0.74, Fig. 5C) and found no variation. The liver is rich in CAT, a major enzyme involved in H_2O_2 detoxification. Then, we investigated if the decrease in hepatic H_2O_2 level could be due to CAT activation. Indeed, we found a 1.5-fold increase in CAT activity in liver of Cbs +/- mice compared to Cbs +/+ mice (100.0±3.8 % vs. 151.8±19.0 %, p=0.008, Fig. 5D). Then, we examined the expression of CAT to determine if the increase in activity was due to an overexpression. However, we did not found any variation of CAT mRNA expression between wild type mice and our hyperhomocysteinemic model (100.0±6.0 % vs. 98.4±7.4 %, p=0.68), indicating that it is not a regulation of catalase activity at the molecular level.

Discussion

A moderate increase in Hcy level is considered as a risk factor for vascular diseases [5-8]. As in vitro studies demonstrated that Hcy leads to both increased ROS production and decreased antioxidant defenses [19,20,47], numerous studies focused on the vascular redox state of animal models. Hepatic metabolism also has a preponderant role on vascular function by regulating circulating cholesterol homeostasis [14-18]. However, only a limited number of studies characterized the hepatic redox state in case of hyperhomocysteinemia. Then we quantified hepatic levels of ROS and RNS and assayed activities of the main antioxidant enzymes in the liver of Cbs +/- mice.

We decided to use a genetic model rather than a nutritional model to avoid Hcy-independent side effects induced by dietary modifications. Among nutrional approaches folate depletion and methionine enrichment are the most commonly used. Dietary models may be directly relevant to study general consequences of hyperhomocysteinemia since nutritional factors are the primary cause of Hcy accumulation in humans. However, as folates are important cofactors in 1-carbon metabolism, folate deficiency can lead to hypomethylation. Furthermore, Symons *et al*. [48], reported that folate depletion induced endothelial cell layer permeability independently of hyperhomocysteinemia. Similarly, Troen *et al.* [49] demonstrated that a high methionine diet induces vascular symptoms that are more strongly linked to the methionine load than to an Hcy accumulation in ApoE-deficient mice. On the other hand, homozygous CBS deficiency induces a decrease in GSH level [50] resulting from the complete blockade of the transsulfuration pathway. Hence, the redox state of Cbs -/- mice might be strongly influenced by GSH depletion. Therefore,

we used Cbs +/- mice, an intermediate model in which Hcy-independent perturbations are minimal, to study the mechanisms of hepatic Hcy toxicity.

Concomitant with overexpression of HO-1, we found an increase in NADPH oxidase activity in the liver of Cbs +/- mice, which is in line with the results of Woo *et al.* in the liver of mice fed a high-methionine diet [26]. NADPH oxidase activation is the key step in the development of oxidative stress via an accumulation of $O_2^{\cdot -}$. However, there was no accumulation of $O_2^{\cdot -}$ in the liver of Cbs +/- mice compared to Cbs +/+ mice. Moreover, we did not found any accumulation in plasma (data not shown) which is consistent with the results obtained by Dayal *et al.* in carotid artery of Cbs +/- mice [51]. Previous results showed that induction of HO-1 suppressed NADPH-oxidase-derived oxidative stress in hyperlipidemic apolipoprotein (E)-deficient mice [52]. $O_2^{\cdot -}$ also reacts rapidly with biological molecules. Then the modest increase in $O_2^{\cdot -}$ production through NADPH oxidase activation might be quenched by an immediate reactivity of $O_2^{\cdot -}$ with lipids, proteins, NO or to an increase in SOD activity. Previous studies associated increased oxidation of lipids and/or proteins with hyperhomocysteinemia due to whether CBS deficiency [53], methionine supplementation [27-28] or folate depletion [30].

To assess the role of Hcy-induced hepatic physiopathology on NO bioavailability, we quantified RNS. As a measure of NO production we quantified nitrites after nitrates reduction in liver homogenates and found no difference between Cbs +/+ and Cbs +/- mice. Once produced by NO synthase, NO can react with $O_2^{\cdot -}$ to form $ONOO^-$ which can notably damage proteins and DNA. We then quantified $ONOO^-$ in the liver of Cbs +/- mice but found no difference compared to Cbs +/+ mice. Moreover, as it has been described in folate deficiency [30] and methionine supplementation [28] there was no activation of SOD in the liver of Cbs +/- mice. The

dismutation of $O_2^{\cdot -}$ by SOD results in the formation of H_2O_2, a less cytotoxic ROS but which can lead to the formation of the highly reactive hydroxyl radical. Against all expectations, we found a 25% decrease in H_2O_2 hepatic concentration in Cbs +/- mice compared to their Cbs +/+ littermates. We then investigated which antioxidant process could account for such a decrease.

Activities of GPx, GRed and GST in the liver of Cbs +/- mice were similar to those observed in Cbs +/+ mice. These results indicate that GSH-dependent detoxification of H_2O_2 or oxidized lipids is not activated by Hcy. This finding is in agreement with the independence of hepatic GSH concentration and heterozygous CBS deficiency previously reported by Likogianni *et al.* [54]. Nevertheless, this is discrepant with previous results obtained with murine models of hyperhomocysteinemia. In heterozygous CBS-deficient mice, Weiss *et al.* reported a 14 % decrease in GPx-1 activity [31]. This difference might arise from the fact that there is a 6.5-fold factor between the folate contents of our diets. In the cell, GPx contribution to H_2O_2 detoxification is much less than that of CAT [55]. Then, we analyzed CAT activity and found a 1.5-fold increase in the liver of Cbs +/- mice compared to Cbs +/+ mice. This increase could explain the decrease in H_2O_2 hepatic content in Cbs +/- mice. We found that the increase in CAT activity was not due to an overexpression. It is known that CAT activity is regulated via modulation of phosphorylations [56]. In this sense, we showed the activation of protein kinase A (PKA) signalling pathways in an *ex vivo* model of hippocampal slices incubated with Hcy [57]. Moreover, Woo et al. also demonstrated in hepatocytes the involvement of PKA signalling pathway in hyperhomocysteinemia [58]. Thus, the activation of PKA by Hcy in hepatocytes could explain the observed increase in CAT activity. Previous studies with dietary approaches observed either a decrease [26,27] or no difference

[28-30] of CAT activity in the liver of Cbs +/- mice compared to Cbs +/+ mice. Such heterogeneity could arise from the fact that different animal models have been used. For instance, a limitation to some studies is that methionine-enriched diet increases GSH levels in wild-type mice which could interfere with Hcy-induced oxidative stress [54].

Our results showed that there was no accumulation of ROS and RNS in the liver of CBS-deficient mice despite an activation of NADPH oxidase. This ability of the liver to maintain its redox balance seems to be due to a significant increase in CAT activity. In aorta of the same model of hyperhomocysteinemia evoked by CBS deficiency, Eberhardt *et al*. [11] reported an increased staining for 3-nitrotyrosine, a marker of peroxynitrite formation, on the endothelial surface. Furthermore, they observed a trend to enhanced lipid peroxidation in serum but not in the liver of CBS-deficient mice. Taken together, their and our findings suggest that Hcy is more deleterious for the vessels than for the liver. This hepatic strength might be due to better antioxidant defenses, as CAT activity is about 10-fold higher in the liver than in the aorta [59,60]. Furthermore, we previously demonstrated that Hcy is intracellular in hepatocytes whereas it is not detected in vascular endothelial and vascular smooth muscle cells [61]. Hence, Hcy toxicity could implicate different pathways depending on whether Hcy is intracellular or extracellular.

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Table 1

Primer sequences for real-time quantitative reverse transcription-polymerase chain reaction.

Gene	Left primer	Right primer
Cat	CCTCCTCGTTCAGGATGTGGTT	CGAGGGTCACGAACTGTGTCAG
Ho-1	GCAGGTGATGCTGACAGAGGAA	GGGGGCCAGTATTGCATTTACA
Sod-1	TGGGGACAATACACAAGGCTGT	TTTCCACCTTTGCCCAAGTCA

All primers are listed 5' to 3'. *Cat*: catalase, *Ho-1*: heme oxygenase-1, *Sod-1*: superoxide dismutase-1.

Figure legends

Fig. 1. Integrated pathways for homocysteine and glutathione synthesis from methionine. DHF, dihydrofolate; DHFR, dihydrofolate reductase; THF, tetrahydrofolate reductase; STHM, serine transhydroxymethylase; CH_2THF, 5,10-methylene tetrahydrofolate; MTHFR, 5,10-methylene tetrahydrofolate reductase; CH_3THF, 5-methyl tetrahydrofolate; MS, methionine synthase; BHMT, betaine homocysteine methyl transferase; DMG, N,N-dimethyl glycine; MAT, methionine adenosyl transferase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; SAHH, S-adenosyl homocysteine hydrolase; CBS, cystathionine beta synthase; CGL, cystathionine gamma lyase; γGCS, γ-glutamyl cysteine synthase; GS, glutathione synthetase; GPx, glutathione peroxidase; GSSG, oxidized glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; G6PD, glucose 6-phosphate dehydrogenase; NADP, oxidized nicotinamide adenine dinucleotide phosphate; GRed, glutathione reductase.

Fig. 2. Effect of CBS genotype on HO-1 expression. HO-1 mRNA levels (A) were quantified by real-time quantitative reverse transcription-polymerase chain reaction. Expression levels are presented as mean percentages ± SEM of mean values of n (number of mice) Cbs +/+ mice. Effect of CBS genotype on HO-1 protein levels has been determined by Western blotting (B). The presented blot specific for HO-1 and β-actin, as a loading control, is representative of 3 experiments with 4 mice in each group. * : $p<0.05$.

Fig. 3. Production and concentration of $O_2^{\cdot -}$ and H_2O_2 in the liver of Cbs +/+ and Cbs +/- mice. Activities and concentrations are presented as mean percentages ± SEM of mean values of n (number of mice) Cbs +/+ mice. * : $p<0.05$.

Fig. 4. Effect of CBS deficiency on RNS hepatic content. Concentrations are presented as mean percentages ± SEM of mean concentration of n (number of mice) Cbs +/+ mice. Nitrites were quantified after nitrates reduction.

Fig. 5. Activities of hydrogen peroxide detoxification enzymes in the liver of Cbs +/+ and Cbs +/- mice. GPx, GRed, GST and CAT activities are presented as mean percentages ± SEM of mean activities of n (number of mice) Cbs +/+ mice. * : $p<0.05$.

Fig 1

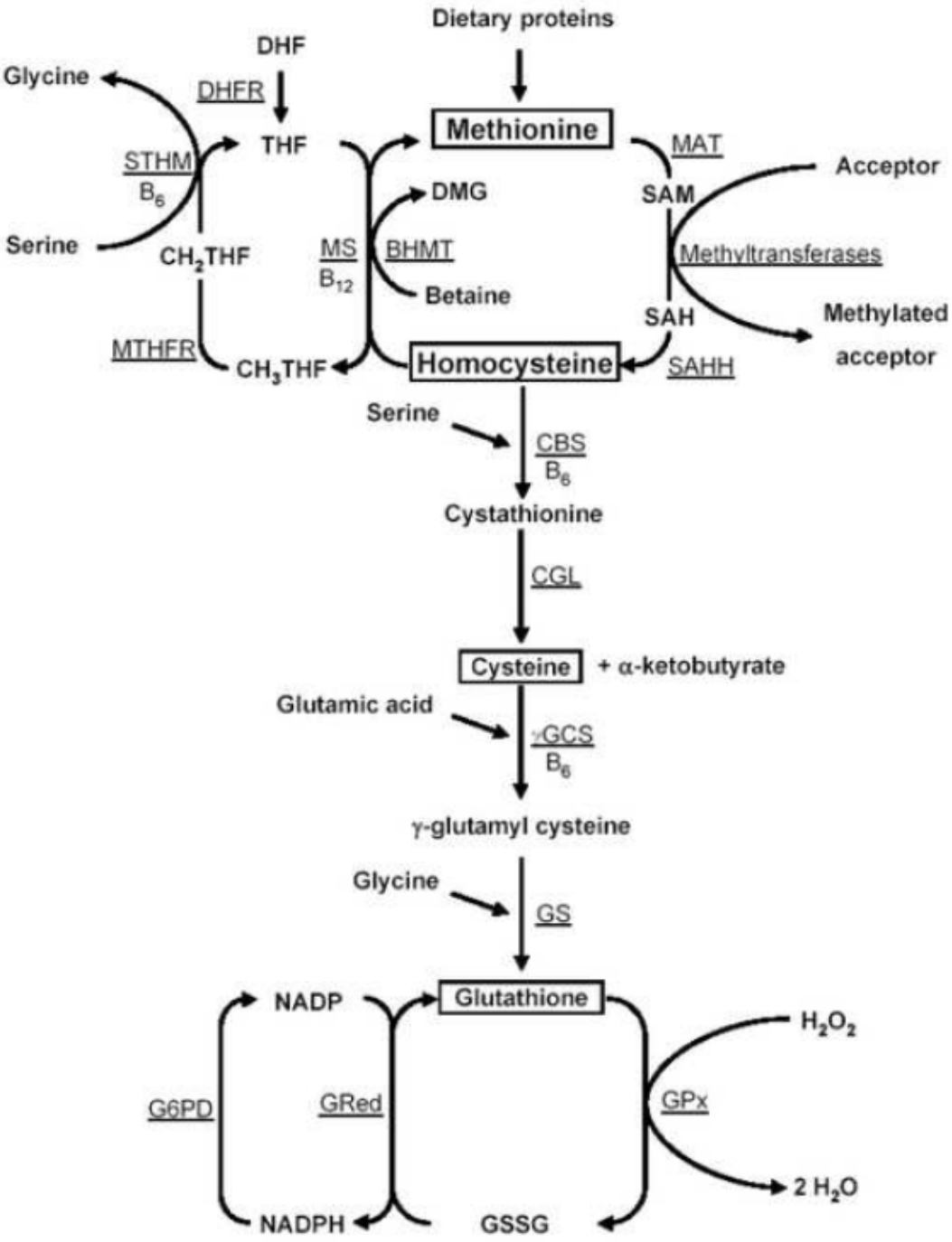

Fig 2

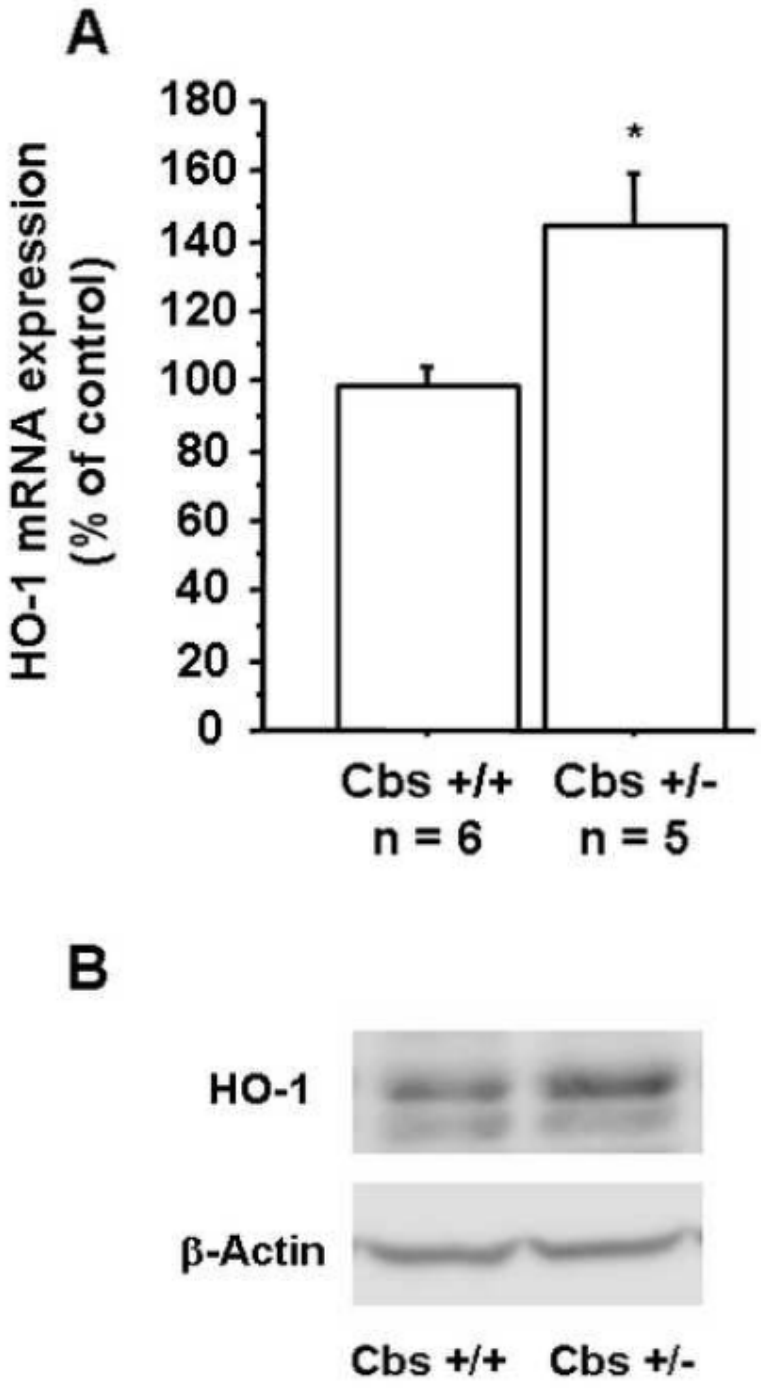

Fig 3

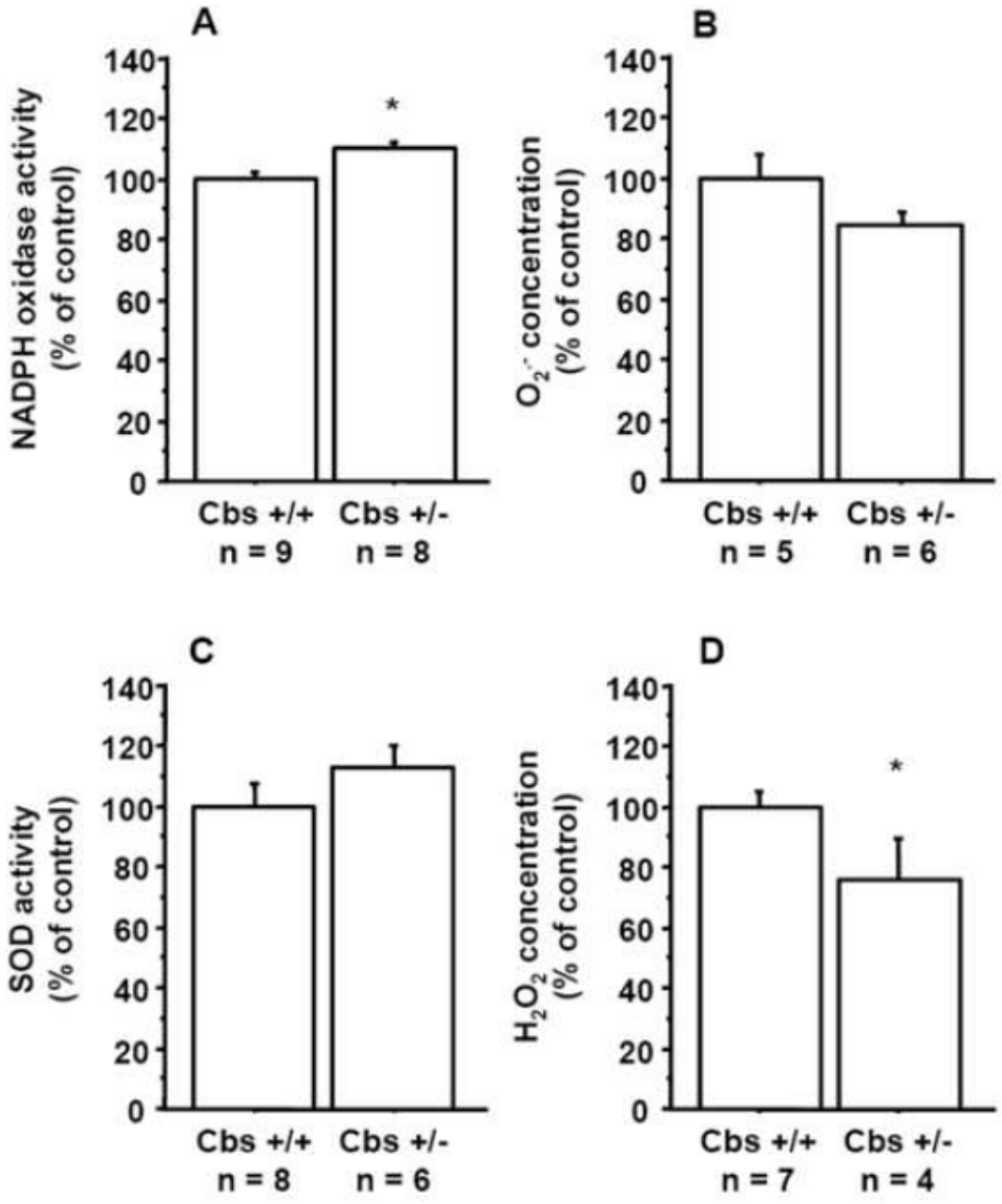

Fig 4

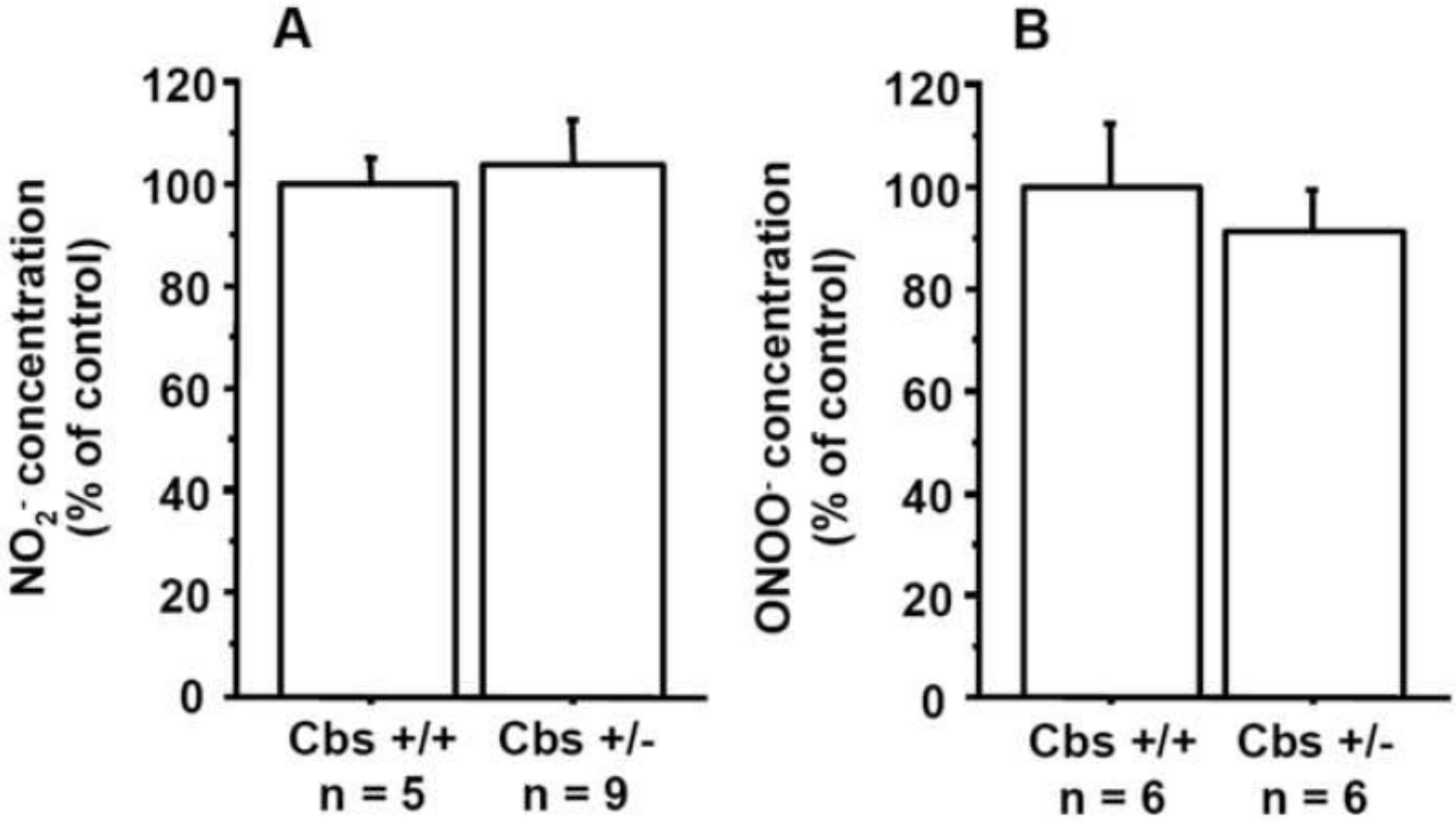

Fig 5